

Chemotaxonomy and flavonoid diversity of *Salvia* L. (Lamiaceae) in Iran

Navaz Kharazian^{1,2}

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ABSTRACT

In this study, we evaluated the chemotaxonomic status and chemical diversity of *Salvia* L. species in Iran using leaf flavonoid profiles. From natural habitats in the country, we collected samples of 14 species of the genus: *S. spinosa* L.; *S. macrosiphon* Boiss.; *S. reuterana* Boiss.; *S. sharifii* Rech.f. & Esfand.; *S. nemorosa* L.; *S. virgata* Jacq.; *S. syriaca* L.; *S. mirzayanii* Rech.f. & Esfand.; *S. atropatana* Bunge; *S. limbata* C. A. Mey; *S. sclarea* L.; *S. ceratophylla* L.; *S. multicaulis* Vahl.; and *S. hydrangea* Dc. ex Benth. Two-dimensional maps of these species were created with thin-layer chromatography. In order to study the taxonomic position of these species and 37 accessions, cluster analysis was applied. The results of the cluster analysis showed that *S. spinosa* was distinct from *S. reuterana*. Despite considerable morphological similarity between *S. nemorosa* and *S. virgata*, those two species are definitely distinguished. In addition, *S. spinosa* and *S. macrosiphon* were roughly grouped, whereas *S. ceratophylla* and *S. multicaulis* composed two separate groups. In the 14 species collected, the flavonoids identified were flavones, flavonols, flavanones, isoflavones, dihydroflavonols and chalcones. We found that flavonoids are appropriate indicators to determine the taxonomic position of *Salvia* species.

Key words: thin-layer chromatography, chemical diversity, *Salvia*, flavonoid, Lamiaceae

Introduction

The genus *Salvia* L. belongs to the Mentheae tribe within the Nepetoideae subfamily of the family Lamiaceae. *Salvia* L. is an important genus, with more than 1000 species worldwide, including 56 species in Iran (Hedge 1982b; Walker *et al.* 2004). It has a cosmopolitan distribution, occurring in arctic, subarctic, temperate, subtropical and tropical areas, including tropical regions of Iran (Hedge 1982b; Walker *et al.* 2004). Some of these species are annual, perennial, herbaceous, suffruticose, fruticose and subshrubby (Hedge 1982b; Khan *et al.* 2002). The main speciation centers of these taxa are considered to be the eastern Mediterranean region; the southwestern, western, eastern and central regions of Asia; Southern Africa; and Central and South America (Hedge 1990; Walker *et al.* 2004; Kahraman & Dogan 2010).

It is known that *Salvia* taxa are used in traditional medicines throughout the world (Anackov *et al.* 2009). The genus has a wide, cosmopolitan distribution and displays a remarkable range of variation (Walker *et al.* 2004). *Salvia* species have been found to have significant biological effects (Sajjadi & Ghannadi 2005), as well as showing high diversity in their secondary metabolites (Flamini *et al.* 2007).

According to the taxonomic and morphologic literature, the sections or groups identified in *Salvia* (Boissier 1879; Hedge 1982b) are not in accordance with each other

(Valant-Vestachera *et al.* 2003). Having such morphological and genomic variability throughout the world, this genus occupies a significant taxonomic position among the plant biosystematics and taxonomy (Baikova 1996). In addition, there is great similarity in morphological characters and considerable hybridization among some *Salvia* species; the genus presents high diversity in terms of polyploid levels and karyotypes (Kharazian 2011); and it is a genus of taxonomic, ecological and genomic complexity. Consequently, the species boundaries have been blurred and no satisfactory classification system yet exists (Valant-Vestachera *et al.* 2003).

Chemotaxonomic studies constitute one of the most important methods of determining the taxonomic positions of taxa. It is now possible to study phenolic profiles of low and high taxonomic levels, even of individual genotypes (Mika *et al.* 2005). Evaluating the patterns of distribution of natural plant products is well established as a major tool for investigating accession structures, species, taxonomic problems and phyletic relationships among genera (Nakiboglu 2002). In addition, flavonoid compounds have been proven to be of chemotaxonomic importance (Fairbrothers *et al.* 1975; Adzet *et al.* 1988; Tomas-Barberan & Wollenweber 1990; Nakiboglu 2002; Valant-Vestachera *et al.* 2003; Kharazian & Rahiminejad 2008, 2009). The *Salvia* genus is a rich source of flavonoid and phenolic acid (Lu & Foo 2000, 2002; Amiri 2007). Consequently, the chemotaxonomy research

¹ Shahrekord University, Faculty of Sciences, Department of Botany, Shahrekord, Iran

² Author for correspondence: nkharazian@gmail.com

of *Salvia* species has focused on their external flavonoid compounds (Adzet *et al.* 1988; Nakiboglu 2002; Lu & Foo 2002; Nikolova *et al.* 2006; Habibvash *et al.* 2007; Gohari *et al.* 2011). In addition, the chemotaxonomic status of the *Salvia* genus has not been exactly determined and needs to be revised in terms of the systematic positions (Nakiboglu 2002). Based on our additional research, using amplified fragment length polymorphism (AFLP) molecular markers, we determined the genetic diversity of the *Salvia* genus in Iran for the first time (Sajadi *et al.* 2010). Valant-Vetschera *et al.* (2003) reported that *Salvia* species show high chemical diversity and largely aggregated flavone composition.

To our knowledge, there have been no chemotaxonomic studies of the various species of *Salvia* in Iran. Because *Salvia* species are distributed in the region, the aim of the present study was to determine the chemotaxonomic status and chemical diversity within the gene pool of the *Salvia* genus. Since some *Salvia* species show great interspecific similarity, and considerable morphological variations, we studied three groups of *Salvia* species: group E—*S. spinosa* L., *S. macrosiphon* Boiss., *S. reuterana* Boiss., *S. sharifii* Rech.f. & Esfand., *S. nemorosa* L., *S. virgata* Jaq., *S. syriaca* L. and *S. mirzayanii* Rech.f. & Esfand.; group D—*S. atropatana* Bunge, *S. limbata* C. A. Mey, *S. sclarea* L., *S. ceratophylla* L.; and group B—*S. hydrangea* Dc. ex Benth. and *S. multicaulis* Vahl. To our knowledge, this is the first report on the chemotaxonomy and chemical diversity of *Salvia* species and accessions in Iran.

Material and methods

Plant material

The locations of the *Salvia* species evaluated from collected material ($n = 14$) and from accessions ($n = 37$), all collected from natural habitats in Iran, are shown in Tab. 1. Voucher specimens were deposited in the Herbarium of Shahrekord University, in the city of Shahrekord, Iran. For each *Salvia* species, we evaluated morphological characters such as leaf, bract, calyx, corolla, style and nutlet features (Tab. 2 and 3).

Sample extraction

The extraction of flavonoids followed the protocol devised by Markham (1982) and Ciesla & Waksmundzka-Hajnos (2010). The flavonoid solution was extracted from air-dried leaves (10.5 g) of 14 *Salvia* species (Tab. 1) using crude 85% MeOH at 60°C. The solvent was removed from the extract with a rotary evaporator at 70°C for total solvent removal and purification of the flavonoids from carotene and chlorophyll was provided using n-BuOH and subsequently analyzed by two-dimensional mapping on silica gel 60F 254 (30 mg, 67.5 ml H₂O) thin-layer chromatography (TLC; 5 µm, 20 × 20 cm). The chromatogram was developed

in BuOH-C₂H₄O₂-H₂O (BAW 3:1:1) representing an organic system. Spot detection with natural product identifiers (5% H₂SO₄/MeOH) was performed under ultraviolet light at 366 nm (Nakiboglu 2002). The presence/absence of spots was taken as the character state and was applied in each species. In addition, we studied the relative mobility (R_f , migration distance of the bands/distance of the solvent front) for each species (Gulen *et al.* 2004). In order to show the taxonomic position of these species, we performed a cluster analysis based on Euclidean distances with Ward's method, focusing on the organic phase and presence and absence of flavonoid spots in TLC profiles, with the Statistical Package for the Social Sciences, version 20.0 (SPSS Inc., Chicago, IL, USA). The purification of flavonoid compounds of each species was carried out with a chromatography column (65 × 3 cm) with sephadex LH20 (Sephadex with 20% MeOH; Sigma-Aldrich, St. Louis, MO, USA) in 100 ml MeOH solution (with graded solutions of 20%, 40%, 60%, 80% and 100% of MeOH and with acetone) and extracted in fractions (the amount of packing material was 50 ml for each level of MeOH content (20%, 40%, 80%, 100%) and for acetone. The fractions were subjected to one-dimensional mapping on silica gels (3 µm). Identification of purified compounds was performed on the basis of their ultraviolet spectra (366 nm), MeOH solution and shift reagents such as AlCl₃, AlCl₃/HCl, NaOAc, NaOAc/H₃BO₃ and MeOH.

Results

The two-dimensional flavonoid patterns of crude extract obtained from each *Salvia* species showed colored spots on chromatography plates. The total numbers of spots obtained for each species and accession were as follows: *S. spinosa*—34, 14 and 9 spots; *S. macrosiphon*—27, 18 and 14 spots; *S. reuterana*—29 and 19 spots; *S. sharifii*—23 and 10 spots; *S. nemorosa*—28, 12 and 11 spots; *S. virgata*—16 and 11 spots; *S. syriaca*—50, 12 and 10 spots; *S. mirzayanii*—22 and 15 spots; *S. multicaulis*—53 and 13 Spots; *S. hydrangea*—57, 20 and 21 spots; *S. atropatana*—34, 15 and 20 spots; *S. limbata*—46, 13 and 15 spots; *S. ceratophylla*—24, 10 and 12 spots; and *S. sclarea*—40, 10 and 11 spots. The color spots detected in 14 *Salvia* species were as follows (Tab. 4 and 5): white-yellow, dark yellow, white-blue, orange, fluorescent yellow, brown, pale violet, fluorescent blue, pale yellow, pale blue, pale orange, yellow-blue, dark brown and yellow-orange.

In some of the species studied, we observed color variations and new color spots after the detection of natural products (Tab. 4 and 5): yellow, violet, pale violet, blue, pale blue, orange, pale orange, brown, dark yellow, pale yellow, white-yellow, yellow-blue, white-blue, fluorescent blue and fluorescent yellow. Those color spots were first reported for *Salvia* species in Iran. The flavonoid classes in the 14 *Salvia* species evaluated are flavones, isoflavones, flavanones, flavonols, dihydroflavonols and chalcones (Tab. 6).

Table 1. The locality of *Salvia* species in natural habitats of Iran.

Species (accession)	Locality	Altitude (m)
<i>S. macrosiphon</i> (119)	Chaharmahal va Bakhtiari - rousa-e Ilbagi	1787
<i>S. macrosiphon</i> (128)	Isfahan - Hojat abad	2189
<i>S. macrosiphon</i> (63)	Fars - Marvdasht	1700
<i>S. spinosa</i> (104)	Chaharmahal va Bakhtiari - Sardabe Rostam abad	1860
<i>S. spinosa</i> (111)	Isfahan - Shams abad	1788
<i>S. spinosa</i> (106)	Tehran - Jajaroud	1250
<i>S. reuterana</i> (27)	Chaharmahal va Bakhtiari - Cheshmeh Sayad	1956
<i>S. reuterana</i> (2)	Isfahan - Semirom	2105
<i>S. syriaca</i> (36)	Lurestan - Khorramabad	2008
<i>S. syriaca</i> (98)	Chaharmahal va Bakhtiari - 30 km from Ardal, Amir abad	1987
<i>S. syriaca</i> (37)	Guilan - Deylaman	1500
<i>S. nemorosa</i> (109)	Chaharmahal va Bakhtiari - Gandoman	1867
<i>S. nemorosa</i> (150)	Chaharmahal va Bakhtiari - Tang-e sayad, Dasht-e chah	1730
<i>S. nemorosa</i> (129)	Isfahan - Naghane, Semirom	1864
<i>S. virgata</i> (78)	Isfahan - Sad-e- Zayanderoud	2310
<i>S. virgata</i> (12)	Chaharmahal va Bakhtiari - Dastgerd	1980
<i>S. sharifii</i> (60)	Isfahan - Kolah Ghazi	1700
<i>S. sharifii</i> (61)	Isfahan - Kolah Ghazi	1660
<i>S. mirzayanii</i> (110)	Fars - Shiraz	1700
<i>S. mirzayanii</i> (2)	Fars - Marvdasht	1850
<i>S. hydrangea</i> (115)	Fars - Abadeh	1800
<i>S. hydrangea</i> (131)	Isfahan - Semirom	1791
<i>S. hydrangea</i> (168)	Chaharmahal va Bakhtiari - Borujen	2000
<i>S. multicaulis</i> (158)	Isfahan - Semirom, Vanak	1950
<i>S. multicaulis</i> (166)	Chaharmahal va Bakhtiari - Do Polan	1659
<i>S. multicaulis</i> (157)	Ahvaz - Izeh	1830
<i>S. ceratophylla</i> (138)	Chaharmahal va Bakhtiari - Bostan Shir	2120
<i>S. ceratophylla</i> (112)	Isfahan - 45 km from Lordegan	1771
<i>S. ceratophylla</i> (137)	Isfahan - Vanak Semirom	1815
<i>S. sclarea</i> (164)	Isfahan -Daran, Damane	1856
<i>S. sclarea</i> (163)	Chaharmahal va Bakhtiari - Naghan, Firouz abad	1988
<i>S. sclarea</i> (165)	Kohgilouye va Boyer Ahmad - Sisakht	1800
<i>S. atropatana</i> (135)	Kurdestan - Marivan	1820
<i>S. atropatana</i> (134)	Isfahan - Semirom	1836
<i>S. atropatana</i> (141)	Chaharmahal va Bakhtiari - Tange Sayad	1836
<i>S. limbata</i> (124)	Chaharmahal va Bakhtiari - Saman, Horeh	2070
<i>S. limbata</i> (123)	Chaharmahal va Bakhtiari - Saman, Ben	

The R_f values in organic solvent systems were obtained for each species profile. As can be seen in Tab. 7, the highest R_f in an organic system ($R_f=2.13$) was found for *Salvia virgata*, whereas the lowest ($R_f=0.01$) was for *S. atropatana*.

In order to determine the taxonomic status of *Salvia* species, cluster analysis was applied using presence and absence of spots.

The cluster analysis based on the presence and absence of flavonoid spots in the TLC profiles of group E produced two groups (Fig. 1), the first comprising *Salvia mirzayanii* and *S. reuterana*, and the second comprising two subgroups: *S. syriaca*, *S. nemorosa*, *S. spinosa* and *S. macrosiphon*; and *S. macrosiphon*, *S. spinosa*, *S. reuterana*, *S. virgata*, *S. sharifii* and *S. nemorosa*. *Salvia virgata* was definitely distinct from *S. nemorosa*. In addition, *S. sharifii* differed from *S. macrosiphon*. *Salvia spinosa* was clearly separate from *S. reuterana*. A high level of chemical diversity was found in *S. spinosa*, *S. reuterana*, *S. macrosiphon*, *S. nemorosa* and *S. syriaca* (Fig. 1 and 2). An additional cluster analysis including four *Salvia* species with high morphological relationships is shown in Fig. 2. The additional cluster analysis also produced two groups, both of which comprised two subgroups. The first group contained *S. sharifii*, *S. reuterana* and *S. macrosiphon* in one subgroup and *S. spinosa* in the other. The second group contained *S. macrosiphon* and *S. spinosa* in one subgroup and *S. reuterana* in the other. This result showed that *S. reuterana* and *S. spinosa* were clearly separated. *Salvia macrosiphon* definitely differed from *S. sharifii*. Furthermore, *S. macrosiphon* and *S. reuterana* were closely related, and the former also clustered with *S. spinosa* (Fig. 2). The cluster analysis of group B and group D also produced two groups, both also comprising two subgroups (Fig. 3). The first group contained *S. ceratophylla*, *S. sclarea* and *S. atropatana* in one subgroup and *S. atropatana*, *S. sclarea* and *S. multicaulis* in the other. The second group contained *S. atropatana* and *S. limbata* in one subgroup and *S. ceratophylla*, *S. hydrangea* and *S. multicaulis* in the

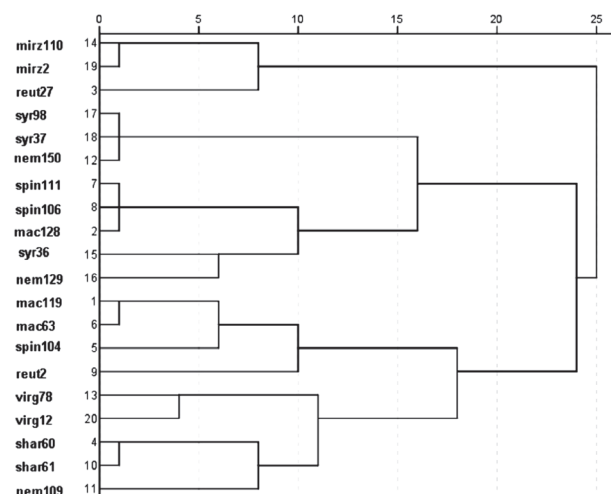
**Figure 1.** Dendrogram of 20 *Salvia* accessions belonging to group E.

Table 2. The morphological characters studied in some *Salvia* species.

Character	<i>S. macrosiphon</i>	<i>S. reuterana</i>	<i>S. spinosa</i>	<i>S. syriaca</i>	<i>S. nemorosa</i>	<i>S. virgata</i>	<i>S. sharifii</i>	<i>S. mirzayanii</i>
Leaf form	Ovate-oblong, elliptic, ovate	Ovate, ovate-oblong	Ovate, broadly elliptic, ovate-oblong, broadly ovate	Ovate, ovate-oblong, ovate-lanceolate	Oblong-lanceolate	Ovate-oblong, broadly ovate, oblong	Ovate	Linear, linear-lanceolate
Leaf base form	Rounded, cordate, sub-cordate	Rounded	Sub-cordate, cuneate, rounded, cordate	Cordate	Cordate, sub-cordate	Cordate, sub-cordate	Rounded	Cuneate
Leaf margin form	Sub-entire, serrate, lobate	Sub-entire, entire	Entire, erose, sub-entire, dentate	Erose, serrulate	Crenate, serrulate	Erose, sub-entire, crenulate, serrate	Sub-entire, serrate	Entire
Leaf apex form	Obtuse, acute, rounded	Obtuse	Acute, obtuse	Acute, obtuse	Acute	Acute, obtuse, rounded	Acute, obtuse	Acute
Bract form	Broadly ovate	Broadly ovate	Broadly ovate	Ovate	Ovate	Ovate	Ovate	Broadly ovate
Bract apex form	Acuminate	Acuminate	Acuminate-spinulose	Acuminate	Acuminate	Acuminate	Acuminate	Acuminate
Bract color	Green-yellow, pink	Green-yellow	Green-yellow, pink, green	Green-white	Violet	Green	Green	Violet, white
Calyx form	Tubular	Broadly tubular	Broadly tubular	Tubular	Tubular-campanulate	Tubular-campanulate	Tubular	Tubular
Corolla tube form	Non-invaginated and non-squamulose	Non-invaginated and non-squamulose	Non-invaginated and non-squamulose	Non-invaginated and non-squamulose	Non-invaginated and non-squamulose	Non-invaginated and non-squamulose	Non-invaginated and non-squamulose	Non-invaginated and non-squamulose
Style apex form	Broad dichotomous	Simple dichotomous	Simple, broad dichotomous	Broad dichotomous	Thin dichotomous	Thin dichotomous	Broad dichotomous	-
Nutlet form	Broad ovoid	Sub-spherical	Rounded-trigonal, spherical	Rounded-trigonal	Ovoid	Ovoid	Ovoid	Ovoid
Nutlet color	Light brown	Light brown	Light brown, dark brown, light gray	Yellow	Black, dark brown	Black, dark brown	Light brown	Black

Table 3. The morphological characters studied in some *Salvia* species.

Character/species	<i>S. multicaulis</i>	<i>S. hydrangea</i>	<i>S. limbata</i>	<i>S. atropatana</i>	<i>S. ceratophylla</i>	<i>S. sclarea</i>
Leaf form	Elliptic, suborbicular	Pinnatisect	Broad ovate, broad cordate	Broad elliptic, oblong, oblanceolate, anguste elliptic	Pinatifida	Ovate, ovate-oblong, obovate
Leaf base form	Cordate, oblique	Narrow	Cordate	Oblique, cuneate, lobed, erose, subentire	Narrow	Cordate
Leaf margin form	Crenulate, rugose	Entire	Erose	Sinuate, crenate, dentate	Lobed	Crenate, erose, lobed
Leaf apex form	Obtuse	Acute	Obtuse, acute	Obtuse	Obtuse	Acute, obtuse
Bract form	Broadly ovate, lobed	Ovate	Broadly ovate, lobed	Broadly ovate	Broadly ovate	Broadly ovate
Bract apex form	Acuminate	Acuminate	Acuminate	Acuminate	Cuspidate	Acuminate
Bract color	Green, pale violet, Pale pink	Green	Green	Green-yellow, green-pink	Green	White-green, pink
Calyx form	Late campanulate	Campanulate-infundibuliformis	Tubular-campanulate, campanulate	Campanulate	Ovate-campanulate	Ovate-campanulate
Corolla tube form	Rectus, annulate	Incompletely annulate or exannulate	Ventricose, squamulose	Ventricose, Squamulose	Ventricose, squamulose	Ventricose, squamulose
Style apex form	Broad dichotomous, simple	Simple dichotomous	Broad dichotomous	Broad dichotomous	Broad dichotomous	Broad dichotomous, simple dichotomous
Nutlet form	Ovoid, rounded-trigonal	Subspherical, rounded-trigonal	Ovoid, spherical	Spherical, ovoid	Spherical	Rounded, rounded-trigonal
Nutlet color	Black, brown, pale brown, creamy	Pale brown	Pale brown	Yellow-cream, brown	Black, violet	Black, pale brown

Table 4. Presence and absence of each spot in *Salvia* accessions before and after detection of natural products.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13
<i>S. macrosiphon</i> (119)	+, +a	+	+, +a	+, +a	-	-	-, +a	-, +a	-	-	-	-	-
<i>S. macrosiphon</i> (128)	-	-	-	+, +a	-	+	-	-	+, +a	-	-	-	+
<i>S. macrosiphon</i> (63)	-	-	-	+	-	+, +a	-	-	-	-	-	-	+
<i>S. spinosa</i> (104)	+, +a	-	+	+	-	+	+	+, +a	+	+	-	-	-
<i>S. spinosa</i> (111)	-	-	+	+	-	-	-	-	-	-	-	-	+, +a
<i>S. spinosa</i> (106)	-	-	+	+	-	-	-	-	-	-	-	-	+
<i>S. reuterana</i> (27)	+, +a	-	+, +a	-	+	-	-	-, +a	-	-, +a	+a	-	-
<i>S. reuterana</i> (2)	+	-	-	+	-	+, +a	+	-	-	-	-	-	-
<i>S. syriaca</i> (36)	+, +a	-	+, +a	+	-	+	+	+	-, +a	+	-	-	-
<i>S. syriaca</i> (98)	-	+	+	-	-	-	-	-	-	-	-	-	-
<i>S. syriaca</i> (37)	-	-	+	-	-	-	-	-	-	-	-	-	-
<i>S. nemorosa</i> (109)	+	-	+	+	+	+	-	-	-	-	-	+a	-
<i>S. nemorosa</i> (150)	-	-	+	-	-	-	-	-	-	-	-	+, +a	-
<i>S. nemorosa</i> (129)	+	-	-	-	-	-	-	-	-	-	-	+	-
<i>S. virgata</i> (78)	+	-, +a	+, +a	+	-	-	-	-, +a	-	-	-	+a	-
<i>S. virgata</i> (12)	+	-	-	+	-	-	-	-	-	-	-	-	-
<i>S. sharifii</i> (60)	+	+, +a	+, +a	+	-	+	-	-	-	-	-	+a	-
<i>S. sharifii</i> (61)	+	-	+	+	-	+	-	-	-	-	-	-	-
<i>S. mirzayanii</i> (110)	+	-	+	-	-	-	-	+	-	-, +a	-	-	-
<i>S. mirzayanii</i> (2)	+	-	+	+	-	-	-	-	-	-	-	-	-

a – the spots after detection of natural product. 1 – yellow, 2 – white-yellow, 3 – blue, 4 – violet, 5 – dark yellow, 6 – white-blue, 7 – orange, 8 – fluorescent yellow, 9 – brown, 10 – fluorescent blue, 11 – pale yellow, 12 – pale blue, 13 – pale violet.

Table 5. Presence and absence of each spot in *Salvia* accessions before and after detection of natural products.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13
<i>S. hydrangea</i> (115)	+	+	+, +a	+	+	+a	-	+	-	+a	-	-	+, +a
<i>S. hydrangea</i> (131)	+	-	+	+	-	-	-	-	-	-	-	-	-
<i>S. hydrangea</i> (168)	+	-	+	-	-	-	-	-	-	-	-	-	-
<i>S. multicaulis</i> (166)	+, +a	+a	+, +a	-	-	+	-	+	-	-	+	-	-
<i>S. multicaulis</i> (157)	+	-	+	-	+	-	-	-	+	-	-	-	-
<i>S. multicaulis</i> (158)	+	-	-	-	+	-	-	-	+	-	-	-	-
<i>S. ceratophylla</i> (138)	+, +a	-	+a	-	-	+	-	-	-	-	-	-	-
<i>S. ceratophylla</i> (112)	+	-	-	+	-	-	-	-	-	-	-	-	-
<i>S. ceratophylla</i> (137)	+	-	+	+	-	-	-	-	+	-	-	-	-
<i>S. sclarea</i> (164)	+, +a	+, +a	+	+a	-	-	+	+	+	-	-	-	-
<i>S. sclarea</i> (163)	+	-	+	-	+	-	-	-	+	-	-	-	-
<i>S. sclarea</i> (165)	-	-	+	+	+	-	-	-	+	-	-	-	-
<i>S. atropatana</i> (135)	+, +a	+, +a	+, +a	-	+, +a	-	-	-	-	-	-	-	-
<i>S. atropatana</i> (134)	+	-	+	+	+	-	-	-	+	-	-	-	-
<i>S. atropatana</i> (141)	+	-	+	+	-	-	-	-	+	-	-	-	-
<i>S. limbata</i> (124)	+	+, +a	+, +a	-	-	+a	+, +a	-	-	-	-	+	-
<i>S. limbata</i> (123)	-	+	+	+	-	-	+	-	+	-	-	-	-

a – the spots after detection of natural product. 1 – yellow; 2 – violet; 3 – blue; 4 – orange; 5 – brown; 6 – dark yellow; 7 – fluorescent yellow; 8 – fluorescent blue; 9 – white-blue; 10 – pale orange; 11 – dark brown; 12 – yellow-orange; 13 – yellow-blue.

Table 6. Flavonoid classes in *Salvia* accessions.

Species	Flavonoid class
<i>S. hydrangea</i>	Flavones, flavanones, flavonols, isoflavones, dihydroflavonols
<i>S. multicaulis</i>	Flavones, flavanones, flavonols, isoflavones, dihydroflavonols, chalcones
<i>S. ceratophylla</i>	Flavones, flavanones, flavonols, isoflavones
<i>S. sclarea</i>	Flavones, flavanones, flavonols, isoflavones, dihydroflavonols, chalcones
<i>S. atropatana</i>	Flavones, flavanones, flavonols, isoflavones, dihydroflavonols, chalcones
<i>S. limbata</i>	Flavones, flavanones, flavonols, isoflavones, chalcones
<i>S. macrosiphon</i>	Flavones, flavonols, flavanones
<i>S. spinosa</i>	Flavones, flavanones
<i>S. reuterana</i>	Flavones, flavanones, flavonols
<i>S. syriaca</i>	Flavones, flavanones
<i>S. nemorosa</i>	Flavones, isoflavones
<i>S. virgata</i>	Flavones, flavonols, isoflavones
<i>S. sharifii</i>	Flavones, flavonols, flavanones, chalcones
<i>S. mirzayanii</i>	Isoflavones

other (Fig. 3). In these results, most of the species displayed chemical diversity. Moreover, *S. multicaulis* and *S. ceratophylla* comprised two separate groups.

Morphological characters—leaf form, leaf margin, leaf base form, leaf apex form, bract apex form, bract color, calyx form, corolla tube form, style apex form, nutlet form and nutlet color—were investigated in 14 *Salvia* species. As can be seen in Tab. 2 and 3, leaf features, bract apex form,

calyx form, style apex form, nutlet form and nutlet color were found to be appropriate morphological characters to differentiate among those 14 *Salvia* species (Kharazian 2012b). *Salvia spinosa* was distinguished from *S. reuterana* by the bract apex form, bract color, leaf margin, leaf base form and nutlet features. *Salvia nemorosa* clearly differed from *S. virgata* in leaf form, leaf margin form, leaf apex form and bract color. In addition, *S. sharifii* and *S. macrosiphon* differed in leaf form, leaf base form, leaf margin and bract form (Tab. 2). It can be concluded that these morphological characters are diagnostic features (Kharazian 2012b).

The taxonomic positions of the 37 *Salvia* accessions and 14 *Salvia* species (group B, D and E) were determined using cluster analysis and identification of spots. The cluster analysis produced two groups, each comprising two subgroups (Fig. 4). The first group contained *S. mirzayanii*, *S. reuterana*, *S. syriaca*, *S. hydrangea*, *S. ceratophylla*, *S. limbata*, *S. atropatana* and *S. multicaulis* in one subgroup and *S. ceratophylla*, *S. sclarea*, *S. atropatana* and *S. multicaulis* in the other. The second group contained *S. nemorosa*, *S. sharifii* and *S. virgata* in one subgroup and *S. spinosa* and *S. macrosiphon* in the other. Among the accessions, the main distinction was between *S. nemorosa* and *S. virgata*. *Salvia spinosa* was clearly distinct from *S. reuterana*. In addition, *S. spinosa* accessions were grouped with *S. macrosiphon* accessions. *Salvia ceratophylla* clustered with *S. hydrangea*, *S. sclarea* and *S. atropatana*. *Salvia limbata* was grouped only with *S. atropatana*. *Salvia atropatana*, *S. multicaulis*, *S. hydrangea*, *S. syriaca*, *S. nemorosa*, *S. macrosiphon* and *S. spinosa* accessions displayed high chemical diversity (Fig. 4).

Based on the patterns of flavonoid variation (Tab. 8 and 9), we observed B-ring ortho-dihydroxylation in *Salvia spinosa*,

Table 7. Relative mobility values for *Salvia* species in the organic solvent system.

Species	R_f value		
	Minimum	Maximum	Mean
<i>S. hydrangea</i>	0.13	1.39	0.88
<i>S. multicaulis</i>	0.13	1.48	0.87
<i>S. ceratophylla</i>	0.11	1.54	1.05
<i>S. sclarea</i>	0.33	1.34	0.84
<i>S. atropatana</i>	0.01	0.94	0.52
<i>S. limbata</i>	0.31	1.31	0.73
<i>S. macrosiphon</i>	0.16	1	0.61
<i>S. spinosa</i>	0.15	1.19	0.77
<i>S. reuterana</i>	0.24	1.37	0.75
<i>S. syriaca</i>	0.42	1.08	0.78
<i>S. nemorosa</i>	0.11	1.25	0.71
<i>S. virgata</i>	0.69	2.13	1.39
<i>S. sharifii</i>	0.59	1.29	1.03
<i>S. mirzayanii</i>	0.15	1.12	0.77

R_f – relative mobility (migration distance of the bands/distance of the solvent front).

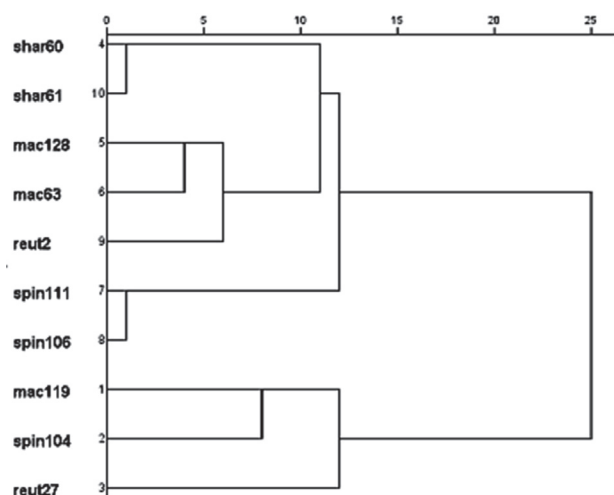


Figure 2. Dendrogram of 10 *Salvia* accessions of *Salvia spinosa*, *S. reuterana*, *S. macrosiphon* and *S. sharifii*.

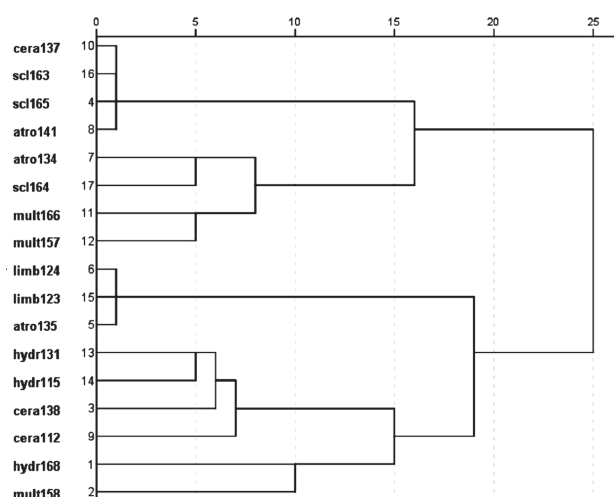


Figure 3. Dendrogram of 17 *Salvia* accessions belonging to group B and group D.

S. macrosiphon, *S. reuterana*, *S. syriaca*, *S. mirzayanii*, *S. nemorosa*, *S. ceratophylla*, *S. sclarea* and *S. limbata*, whereas we observed A-ring ortho-dihydroxylation in *S. spinosa*, *S. reuterana*, *S. mirzayanii*, *S. syriaca*, *S. nemorosa*, *S. hydrangea*, *S. multicaulis*, *S. sclarea*, *S. atropatana* and *S. limbata*. In most of the *Salvia* species, there was a tendency toward 3-hydroxylation, 5-hydroxylation, 7-hydroxylation, 2'-hydroxylation, 3'-hydroxylation, 4'-hydroxylation and 4'-methoxylation. In some species, 2-hydroxylation, 4-hydroxylation, 6-hydroxylation, 8-hydroxylation, 5'-hydroxylation, 5-methoxylation, 6-methoxylation, 7-methoxylation, 8-methoxylation, 2'-methoxylation and 3'-methoxylation were present (Tab. 8 and 9). We also observed other substitutions (Tab. 8 and 9), including 7-*o*-rhamnoglucosylation; 3-*o*-glucosylation; 5-*o*-glucosylation; 7-*o*-glucosylation; 7-*o*-rhamnosylation; 3-*o*-rhamnuogalactosylation; 7-*o*-glucuronosylation; 3-*o*-rhamnosylation; 3-*o*-galactosylation; 8-*c*-

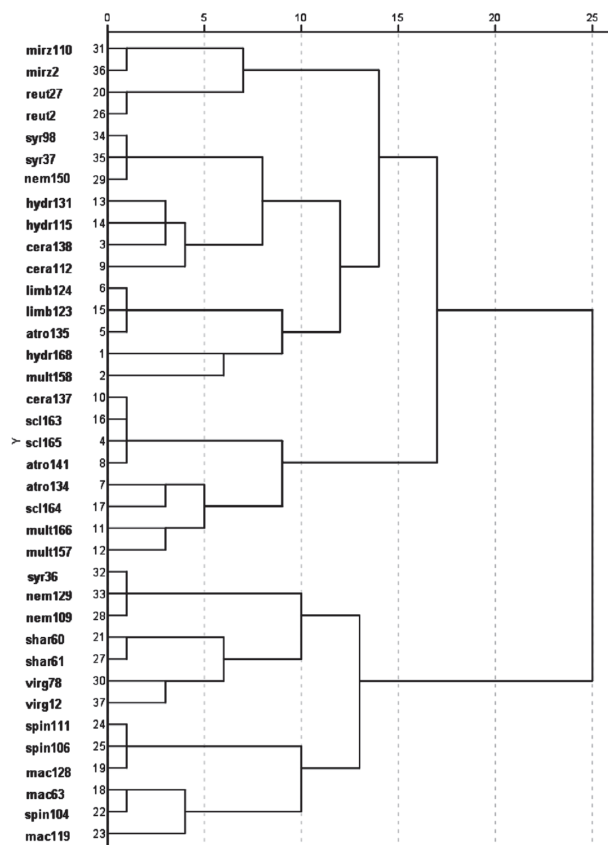


Figure 4. Dendrogram of 37 *Salvia* accessions belonging to group B, group D and group E.

rhamnoglucosylation; 8-*c*-glucosylation; 7-*o*-rutinosylation; 6-*c*-glucosylation; 3-, 3'- and 4'-methylene dioxydization; 3-*o*- β -glucopyranosylation; and 2-carboxylation. This is the first report of flavonoid variations for *Salvia* species in Iran.

Discussion

According to the taxonomic literature, *Salvia* species are divided into five groups by leaf form, stamen type, corolla tube and calyx form (Hedge 1982b). *Salvia syriaca*, *S. nemorosa*, *S. reuterana*, *S. macrosiphon*, *S. spinosa*, *S. virgata*, *S. sharifii* and *S. mirzayanii* constitute a group in which the corolla tube is not invaginated or squamulose and the inside of the corolla tube is imperfectly or perfectly annulated (Hedge 1982b). Morphologically, *Salvia reuterana*, *S. spinosa* and *S. macrosiphon* are closely related. In the present study, the number of spots differ among *S. reuterana*, *S. spinosa* and *S. macrosiphon* (29 and 19; 34, 14 and 9; and 27, 18 and 14 spots, respectively). In terms of the R_f values in the organic phase, *S. spinosa* differed from the two other species, which is in agreement with Sajadi *et al.* (2010). In addition, in the TLC profile of *S. spinosa*, white-blue and brown spots were observed. *Salvia spinosa* is a polymorphic taxon with high morphological variability

Table 8. Flavonoid variation patterns in *Salvia* species.

Variation pattern	sp	mac	reut	syr	nem	virg	shar	mirz
A-ring ortho-dihydroxylation	+	-	+	+	+	-	-	+
B-ring ortho-dihydroxylation	+	+	+	+	+	-	-	+
2-hydroxylation	-	+	-	-	+	-	+	-
3-hydroxylation	+	+	-	-	+	+	+	+
5-hydroxylation	+	+	+	+	+	+	+	+
6-hydroxylation	+	-	+	-	+	-	-	+
7-hydroxylation	+	+	+	+	+	+	+	+
8-hydroxylation	-	+	-	+	+	+	+	+
2'-hydroxylation	-	+	+	-	+	-	+	+
3'-hydroxylation	+	+	+	+	+	+	+	+
4'-hydroxylation	+	+	+	+	+	+	+	+
5'-hydroxylation	-	-	-	-	+	-	-	-
5-methoxylation	-	+	+	-	+	-	-	-
6-methoxylation	-	+	+	-	+	+	+	+
7-methoxylation	-	+	+	+	+	+	+	+
8-methoxylation	-	-	-	-	-	-	-	+
2'-methoxylation	+	-	-	-	-	-	-	-
3'-methoxylation	-	-	-	-	+	+	-	+
4'-methoxylation	+	+	+	+	+	+	+	+
7- <i>o</i> -rhamnoglucosylation	+	-	+	-	+	+	-	-
3'-methylene dioxidization	-	-	-	-	-	+	-	+
4'-methylene dioxidization	-	-	-	-	-	+	-	+
3- <i>o</i> -glucosylation	-	+	+	-	-	-	+	+
5- <i>o</i> -glucosylation	-	-	-	+	+	-	-	-
7- <i>o</i> -glucosylation	-	-	-	+	-	+	-	+
8- <i>c</i> -glucosylation	-	-	-	-	-	+	-	-
6- <i>c</i> -glucosylation	-	-	-	+	-	-	-	+
7- <i>o</i> -rhamnosylation	-	-	+	-	+	-	-	-
3- <i>o</i> -rhamnosylation	-	-	-	-	+	-	-	-
3- <i>o</i> -rhamnogalactosylation	-	-	+	-	-	-	-	-
7- <i>o</i> -glucuronosylation	-	-	+	+	-	-	-	-
3- <i>o</i> -galactosylation	-	-	-	-	+	-	-	-
8- <i>c</i> -rhamnoglucosylation	-	-	-	-	+	-	-	-
7- <i>o</i> -rutinosylation	-	-	-	-	-	+	-	-
2-carboxylation	+	-	+	-	-	-	-	-

sp – *Salvia spinosa*; mac – *S. macrosiphon*; reut – *S. reuterana*; syr – *S. syriaca*; nem – *S. nemorosa*; virg – *S. virgata*; shar – *S. sharifii*; mirz – *S. mirzayanii*.

(Kharazian 2009; 2012b). Furthermore, some of the *S. reuterana* accessions from the Azerbaijan province are similar to *S. spinosa* in term of the calyx in the fruit (Hedge 1982b). *Salvia spinosa* includes morphological characters that are not easily distinguishable from those of *S. reuterana*. In the present study, the flavonoid profiles, flavonoid types and morphological features were found to be appropriate markers to discriminate the two species. The main morphological characters differentiating *S. spinosa* from *S. reuterana* are

the bract features (Kharazian 2009). In the present study, *S. reuterana* was distinguished by its flavonol compounds. Conversely, some of the *S. spinosa* accessions from Turkey are similar to those of *S. macrosiphon* from Iran and Afghanistan (Hedge 1982a; Kahraman *et al.* 2009), which are closely grouped using flavonoid profiles. Kharazian (2012b) also showed that *S. spinosa* and *S. macrosiphon* were closely related morphologically but differs in diagnostic characters such as the form of the leaves and calyx.

Table 9. Flavonoid variation patterns in *Salvia* species.

Variation pattern	hydr	mult	cera	scl	atro	limb
A-ring ortho-dihydroxylation	+	+	-	+	+	+
B-ring ortho-dihydroxylation	-	-	+	+	-	+
2-hydroxylation	-	-	-	-	-	+
3-hydroxylation	+	+	+	+	+	+
4-hydroxylation	-	+	-	+	+	-
5-hydroxylation	+	+	+	+	+	+
6-hydroxylation	+	+	-	+	-	+
7-hydroxylation	+	+	+	+	+	+
8-hydroxylation	+	+	-	-	-	+
2'-hydroxylation	+	+	+	+	+	+
3'-hydroxylation	+	+	+	+	+	+
4'-hydroxylation	+	+	+	+	+	+
5'-hydroxylation	+	-	-	-	-	-
6-methoxylation	+	+	+	+	+	+
7-methoxylation	-	+	+	-	-	+
8-methoxylation	+	+	+	+	+	+
3'-methoxylation	+	+	+	-	+	+
4'-methoxylation	+	+	+	+	+	+
7- <i>o</i> -rhamnoglucosylation	-	+	-	-	-	+
3-methylene dioxidization	-	-	+	+	-	+
3'-methylene dioxidization	+	+	+	-	+	+
5- <i>o</i> -glucosylation	-	-	-	-	-	+
8- <i>c</i> -glucosylation	-	-	+	-	-	-
6- <i>c</i> -glucosylation	-	-	+	-	-	-
3- <i>o</i> -glucosylation	-	+	-	+	+	-
7- <i>o</i> -glucosylation	-	-	-	-	+	+
7- <i>o</i> -glucuronosylation	+	+	-	+	+	+
3- <i>o</i> -galactosylation	-	+	-	-	-	-
3- <i>o</i> - β -glucopyranosylation	-	-	+	-	-	+

hydr – *Salvia hydrangea*; mult – *S. multicaulis*; cera – *S. ceratophylla*; scl – *S. sclarea*; atro – *S. atropatana*; limb – *S. limbata*.

In our cluster analysis, *Salvia reuterana* and *S. macrosiphon* were in separate groups. This differentiation was not observed by Sajadi *et al.* (2010). Salimpour *et al.* (2011), using essential oil composition, found that these two species were different, which is in agreement with our results. It can be concluded that environmental conditions and forms of secondary metabolites contribute to the different results obtained with molecular markers (Maksimovic *et al.* 2007).

In the cluster analysis, *Salvia syriaca* was grouped with *S. nemorosa*. Nevertheless, one *S. syriaca* accession was quite different from the other species in term of the number of color spots. In the flora of Turkey and Russia, *S. syriaca* has been mentioned in a separate group (Pobedimova 1954; Hedge 1982a). In our results, using the AFLP molecular marker, Sajadi *et al.* (2010) also reported that *S. syriaca* accessions were grouped with *S. nemorosa*, which is in

agreement with Bagcia *et al.* (2004), Goren *et al.* (2006) and Kharazian (2012b). It can be concluded that *S. syriaca* accessions showed high chemical diversity. In addition, our results are in accordance with the findings of Bagcia *et al.* (2004), Goren *et al.* (2006) and Habibvash *et al.* (2007), who used fatty acid compositions and phenolic compounds. Kharazian (2012b) and Sajadi *et al.* (2010) showed high morphological and molecular diversity in *S. syriaca* accessions.

On the basis of our data, *Salvia nemorosa* was grouped with other species, which is correlated with the hybridization of *S. nemorosa* with different species (Hedge 1982a, 1982b; Sajadi *et al.* 2010). Janicsak *et al.* (2006) reported the variability in oleanolic and ursolic acid contents among subspecies of *S. nemorosa*. In addition, *S. nemorosa* is clearly separate from *S. virgata*, which is based on Sajadi *et al.* (2010). Furthermore, in the flora of Russia, these two species were

mentioned in two separate series (Pobedimova 1954). Notably, these two species have a high morphological similarity, which makes it difficult to separate the two. As was observed in the present study, the two species are distinguished by bract color and leaf features (Kharazian 2012b). Evidently, in the organic phase, *S. virgata* is a separate species, which is based on Goren *et al.* (2006). Evaluating nutlet anatomy, Habibvash & Rajamand (2007) reported that *S. virgata* and *S. nemorosa* were distinct. Moreover, nutlet morphology in *S. virgata* showed that the apomorphic characteristic of this species distinguishes it from all other *Salvia* taxa (Ozkan *et al.* 2009). Tosun *et al.* (2009) also mentioned that these two species showed significant differences in antioxidant activity and total phenolic compounds. It can be concluded that the organic phase is appropriate and can be well documented. In our study, it seemed that flavonol compounds differentiated these two species. The presence of fluorescent yellow in *S. virgata* is supported by the chemotaxonomy results obtained by Nakiboglu (2002). Notably, Nakiboglu (2002) reported only four spots for this species, which is not in accordance with our findings (16 and 11 spots).

In the cluster analysis, *Salvia sharifii* and *S. macrosiphon* were grouped separately (Sajadi *et al.* 2010). Some of the *S. sharifii* accessions from the southern and southeastern regions of Iran have been associated with *S. macrosiphon* (Hedge 1982b), which is not supported our results. It seems that the bract form and leaf features are one of the main morphological characters for separating these two species (Kharazian 2012b). In addition, the two species differed in terms of the flavonoid classes presented.

The results of our cluster analysis in 10 *Salvia* accessions, including *S. macrosiphon*, *S. reuterana*, *S. spinosa* and *S. sharifii*, show that these species are clearly related, which is in agreement with the results of Kharazian (2009) and Sajadi *et al.* (2010). It can be concluded that the presence and absence of flavonoid spots was a significant factor in determining the taxonomic status of *Salvia* species.

The cluster analyses of *Salvia* accessions showed high chemical diversity in *S. spinosa*, *S. syriaca*, *S. reuterana*, *S. nemorosa* and *S. macrosiphon*. Owing to the hybridization and introgression of *S. macrosiphon* with other species, such as *S. moorcroftiana* Wall. Ex Benth. and *S. reuterana*, there are also patterns of variation in *S. macrosiphon* (Hedge 1982a; 1990). Based on the results obtained by Kharazian (2009; 2012b), most of those variations were in leaf indumentum, leaf form, leaf base, leaf margin, bract indumentum, corolla color, corolla tube length, calyx length, calyx apex form and inflorescence indumentum. In addition, Kharazian (2012b) mentioned that the morphological variations in *S. spinosa* accessions were in leaf form, leaf margin form, leaf indumentum, stem indumentum, inflorescence indumentum, bract indumentum, bract dimension, bract color, calyx indumentum, calyx dimension, corolla indumentum and corolla length. Conversely, *S. virgata*, *S. sharifii* and *S. mirzayanii* accessions displayed no chemical diversity.

It has been reported that the variability in flavonoid patterns is influenced by ecological conditions (Tomas-Barberan & Wollenweber 1990).

Salvia multicaulis, *S. hydrangea* and *S. ceratophylla* were placed in a group of species with pinnatisect leaves, and *S. multicaulis* also belongs to a group in which the anthers have lower thecae and the calyx in the fruit is expanded and membranous-reticulate. *Salvia atropatana*, *S. ceratophylla*, *S. sclarea* and *S. limbata* were included in a group of species with invaginated, squamulose corolla tubes, the inside of which is glabrous. In the cluster analysis of these groups, *S. multicaulis* was included in two groups and was clustered with *S. hydrangea*. Kharazian (2012b) reported that the morphological variations in *S. multicaulis* are generally related to the indumentum of the stem and leaf; petiole; calyx and inflorescence axis; leaf form; calyx apex; calyx color; and bract form. It appears that the morphological variations in *S. multicaulis* are closely related to the varieties, forms or polymorphism characters of the species. Sajadi *et al.* (2010) also reported molecular variations among the *S. multicaulis* accessions. *Salvia ceratophylla* accessions were grouped with those of *S. sclarea*, *S. atropatana* and *S. hydrangea*. Sajadi *et al.* (2010) and Kharazian (2012b) reported that *S. ceratophylla* accessions were mostly grouped with *S. sclarea*. In addition, Habibvash *et al.* (2007) reported that *S. ceratophylla* and *S. sclarea* are similar in terms of their contents of linolenic and arachidic acid, which is in keeping with our results. In the present study, *S. ceratophylla* was also included in two groups. We found that *S. atropatana* was clustered with *S. sclarea*, *S. limbata* and *S. ceratophylla*. Using fatty acid, Habibvash *et al.* (2007) reported this relationship in *S. limbata*, *S. sclarea* and *S. ceratophylla*. According to Kharazian (2012a; 2012b), *S. atropatana* displays considerable variation in morphological characters such as leaf form, leaf margin form, leaf indumentum, bract indumentum, calyx indumentum, corolla indumentum and style length. We also found flavonoid variations in *S. atropatana*. *Salvia limbata* accessions were grouped with *S. atropatana*, which is in agreement with Sajadi *et al.* (2010) and Kharazian (2012b). Notably, the two species presented similar classes of flavonoids. We observed flavonoid variations in all of the *Salvia* species belonging to group D. It has been noted that some accessions of *S. sclarea* were included in different groups, which is probably due to morphological variations or ecological adaptations (Kharazian 2012b; Ozdemir & Senel 1999). It seems that these taxonomic differentiations were due to polymorphism in the morphological characters, hybridization between species and geographical distribution. *Salvia sclarea* shows variability in terms of the bract color, bract length, color of the upper lip of the corolla, leaf form and leaf margin (Kharazian 2012b). It seems that flavonoid diversity and its variation is related to morphological variability and to the geographical conditions in Iran.

Flavones, flavanones and flavonols were common in all of the group studied. The species belonging to group E might be separated from two other group using dihydrofla-

vonols. In addition, most of the chalcones and isoflavones were observed in group B and D. It might be concluded that, based on the flavonoid compounds, group B and D were closely related.

On the basis of spot colors, some of the species evaluated have been found to contain isoflavones and flavonols (Tomas-Barberan & Wollenweber 1990; Lu & Foo 2002; Amiri 2007). It seems that these compounds were flavones 7-*o*-rhamnoglucoside; flavones 5-*o*-glycosides; 5-OH flavanones, flavone 3-*o*-glucoside, flavones, flavonol, 5-hydroxyflavonol, isoflavone, flavanone, 5-hydroxyflavanone and dihydroflavonol. In the above cases, the variations in flavonoid patterns of hydroxylation, methoxylation, *o*-glucosylation, *o*-glycosylation and *o*-rhamnoglucosylation, *o*-rhamnosylation, *o*-rhamnogalactosylation, *c*-rhamnoglucosylation, *o*-rutinosylation, *o*-galactosylation, *o*-glucuronosylation, *c*-glucosylation, methylene dioxidization and β -glucopyranosylation occurred in these species which is roughly in accordance with Tomas-Barberan & Wollenweber (1990) and Lu & Foo (2002). Consequently, the widest range of flavonoid variations was found in Grex E. It seems that the variations of flavonoid patterns in 14 *Salvia* species could accurately resolve the taxonomic status.

Conclusion

In conclusion, we can state that flavonoid profiles, as identified through chemotaxonomic studies, appear to be appropriate markers of the taxonomic status of *Salvia* species (Gohari *et al.* 2011). It can be assumed that the variability observed in *Salvia* species arose due to adaptation, to alterations to the reproductive system in response to adverse environmental conditions and to recombination (Haque 1983; Wang *et al.* 2007; Baran *et al.* 2008; Aktas *et al.* 2009). In addition, there is a correlation between the habitat in which the plant grows and the production of flavonoid compounds (Tomas-Barberan & Wollenweber 1990). The high incidence of flavonoid compounds in the *Salvia* genus largely consisted of few taxonomic units. To use flavonoid profiles more widely as genetic markers, they would have to be abundant and expedient, in order to identify the taxonomic position (Fairbrothers *et al.* 1975; Mika *et al.* 2005).

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